AD_____

Award Number: DAMD17-01-1-0557

TITLE: The Role of Stat3 in Breast Cancer Tumorigenesis

PRINCIPAL INVESTIGATOR: Jacqueline F. Bromberg, M.D., Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
New York, New York 10021

REPORT DATE: September 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Final (20 Aug 01 - 19 Aug 02)

4. TITLE AND SUBTITLE
The Role of Stat3 in Breast Cancer Tumorigenesis

5. FUNDING NUMBERS
DAMD17-01-1-0557

6. AUTHOR(S):
Jacqueline F. Bromberg, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sloan-Kettering Institute for Cancer Research
New York, New York 10021

E-Mail: bromberj@mskcc.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

In a growing number of cancers and cancer derived cell lines, where growth factor dysregulation is often at the heart of transformation, Stat3 (one of the STAT proteins) is constitutively activated. In a number of cancer derived cell lines, constitutively activated Stat3 is either required for transformation, enhances transformation or blocks apoptosis. Introduction of either anti-sense or dominant negative Stat3 in a number of cancer derived cell lines including breast cancer leads to their apoptosis. We have engineered a constitutively dimerizeable Stat3, termed Stat3-C, which is capable of inducing cell transformation of immortalized breast epithelial cells, thus qualifying activated Stat3 as a proto-oncogene. The purpose of the Concept Proposal (DAMD17-01-1-0557) was to 1) Identify by both biochemical and immunohistochemical techniques the abundance and distribution of phospho-Stat3 in primary breast cancer samples. We determined that unless the primary sample was "processed" within 30 minutes of removal from the patient, that the levels of phospho-Stat3 decreased substantially. Analysis of archival paraffin tissue sections gave mixed results regarding the strength of the phospho-Stat3 signal. However, tissue arrays gave the most reproducible results and revealed that ~ 35% of primary breast cancer samples contained high levels of phospho-Stat3. A positive correlation with ER and PR but an inverse correlation with her2neu was observed in those samples expressing strong p-Stat3. We examined levels of the Stat3 negative regulator, PIAS3 mRNA in matched normal versus tumour samples and determined (to our surprise) that PIAS3 levels were higher in the tumors containing phospho-Stat3. We generated MMTV Stat3-C mice in collaboration with Robert Schriber. Unfortunately, Stat3-C expression is exceedingly low and the animals do not have any unusual phenotype.

14. SUBJECT TERMS	15. NUMBER OF PAGES
stats, breast carcinogenesis, immunohistochemistry, breast cancer	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....8

Reportable Outcomes.....9

Conclusions.....9

References.....9

Appendices.....

Introduction

Signal Transducers and Activators of Transcription (STATs) are a family of transcription factors which are normally "inactive" within the cytoplasm of cells and upon tyrosine phosphorylation become "activated" which leads to the dimerization of two Stat molecules. Dimerized Stats are translocated into the nucleus where they bind DNA and activate transcription. Stat dimers are dephosphorylated within the nucleus and transported back to the cytoplasm (1). Virtually all growth factor receptors, cytokines, and tyrosine kinases lead to the phosphorylation of one or more Stat proteins. In "normal" cells this activation is transient, while in an ever growing number of primary tumors and cancer derived cell lines Stat proteins (in particular Stat3) are constitutively activated (1, 2). A causal association between activated Stat3 and cellular transformation or oncogenesis has been made in a large number of cancer derived cell lines. Specifically, removal of Stat3 by the introduction of a dominant negative Stat3 molecule or anti-sense molecule leads to a reversal of the transformed phenotype, induction of apoptosis , decreased angiogenesis or growth arrest (Figure 1).

Constitutive Activation of STATs in Primary Cancers and Tumor Derived Cell Lines

Solid Tumors	Activated STAT	Liquid Tumors	Activated STAT
Breast Cancer	1,3	Chronic Myelogenous Leukemia (CML)	5
Head and Neck	1,3	Acute Myeloid Leukemia (AML)	1,3,5
Cancer	3	Chronic Lymphocytic Leukemia (CLL)	1,3
Prostate Cancer	3	Mycosis Fungoides (MF)	3
Melanoma	3	Acute Lymphoblastic Leukemia (ALL)	1,5
Ovarian Cancer	1,3	Erythroleukemia	1,5
Lung Cancer	1,3	Burkitt's lymphoma	3
Brain Tumors	3	Large granular lymphocyte (LGL)	3
Pancreatic	3	leukemia	3
		Myeloma	3
		Hodgkins Lymphoma	3

STAT Activation Required for Transformation

Cell Lines/Primary Tumor	Required STAT	Oncogenes	Required STAT
Breast Cancer	3	src	3
Head and Neck Cancer	3	eyk	3
Prostate Cancer	3	ret	3
Melanoma	3	lck	3,5
Thyroid Cancer	3	Galphao	3
Myeloma	3	Npm-alk	3
Hodgkins	3		
LGL	3		
HCC	3		

Figure 1. Many primary tumors contain constitutively activated Stat3, 5 and 1. Stat3 is required for transformation in the above mentioned tumors as determined by introduction of dominant negative Stat3 molecules and observing growth arrest and or apoptosis.

It has been determined that Stat3 is constitutively activated in primary breast cancer samples, and in a number of breast cancer derived cell lines (Figure 2) (3).

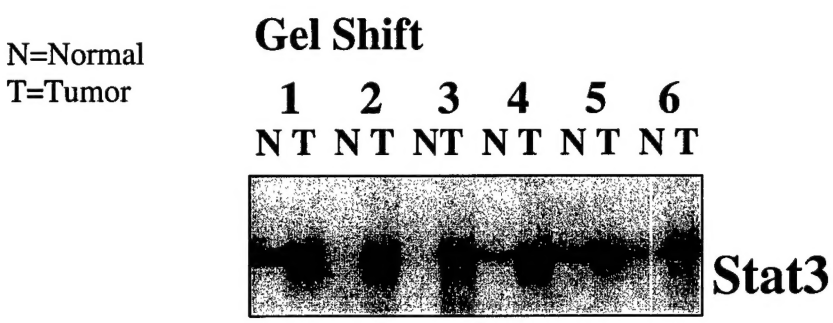

Figure 2. Primary breast cancer samples contain higher levels of Stat3 binding activity in comparison to matched normal tissue.

Furthermore, expression of a dominant negative Stat3 in one of these cell lines (MDA-MB468) promotes its apoptosis(4). In studies performed in rodent fibroblasts, we have demonstrated both the requirement and sufficiency of activated Stat3 in mediating cellular transformation (5, 6, 7). Specifically, we designed a constitutively active mutant form of Stat3 which is dimerized by cysteine-cysteine residues instead of pY-SH2 interactions which can transform immortalized cultured fibroblasts. The use of primary human mammary epithelial (HMEC) cells and immortalized HMEC cell lines are an established *in vitro* model system for studying breast epithelial cell growth and malignant transformation. As it was determined in rodent fibroblasts, we have successfully introduced Stat3-C into 2 different immortalized HMEC derived cell lines (MCF-10A and HMLER). The Stat3-C complemented cells are transformed as determined by anchorage independent growth (our unpublished data).

Given the prevalence of activated Stat3 in primary breast cancer derived cell lines and the sufficiency of activated Stat3 to mediate cellular transformation of rodent fibroblasts, we proposed examining the specific role of constitutively activated Stat3 in breast carcinogenesis, including the prevalence of activated Stat3 in primary breast cancer samples, the abundance and activity of regulators of Stat3 and in transgenic models of breast cancer.

Body

Here we demonstrate that in matched tumor and normal tissue Stat3 is predominantly activated in the tumor samples and not in the normal samples (Figure 2). We went on to develop an immunohistochemical approach to defining the abundance and distribution of activated Stat3 in primary breast cancer samples. We initially observed variable results with archived paraffin sections of breast cancer specimens and suspected that depending on the length of time the primary specimen was left "un-processed" might lead to differences in the phospho-Stat3 signal. In order to address this concern, we obtained fresh samples from the operating room and froze a portion of the sample immediately keeping the remainder on ice for one hour after which time we froze the sample in liquid nitrogen and processed both for analysis of phospho-Stat3 by

western blot analysis and immunohistochemistry. Our results demonstrate that the phospho-Stat3 signal is significantly reduced in the samples which were kept on ice for one hour, both by western blot analysis and immunohistochemistry (Figure 3). Interestingly, the intensity of staining for total Stat3 was also reduced. Thus, when analyzing archival sections we first analyzed the total Stat3 signal and if it was reasonably strong we went on to analyze the samples for phospho-Stat3.

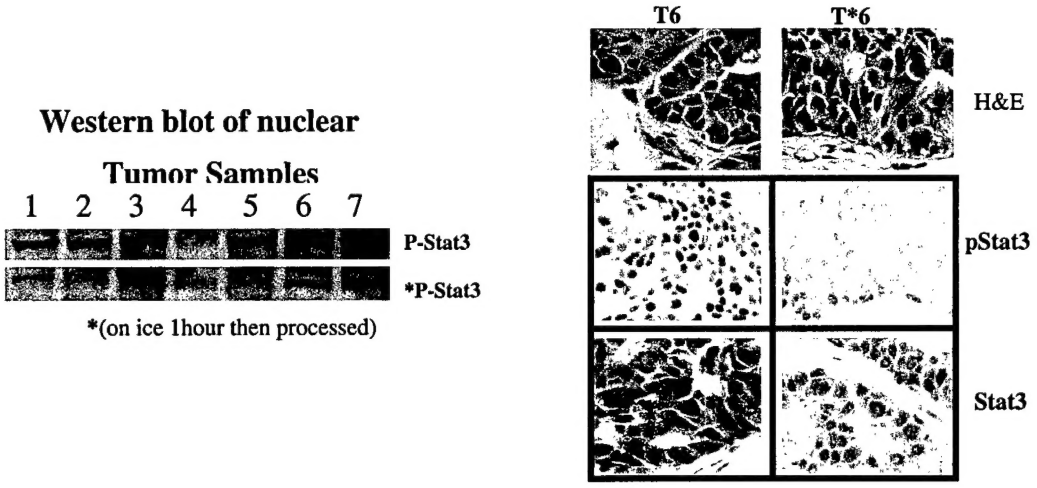

Figure 3. Phospho-Stat3 signal is significantly reduced in tumor samples when not processed within one hour from surgery as determined by western blot analysis as well as by immunohistochemistry

We analyzed individual samples (120) and 26 core biopsy samples (tissue arrays) for the abundance and distribution of activated Stat3. Total Stat (both the phosphorylated and unphosphorylated) is highly expressed in normal cells as well as cancerous cells (Figure 4), which is why an antibody specific for the activated or phospho-Stat3 was utilized in determining where activated Stat3 was localized. Essentially, we determined that 35% of the samples stained strongly for pStat3, 30% stained moderately for pStat3 and 35% did not stain at all for pStat3 cells (Figure5). We then correlated the distribution of activated Stat3 with the expression of known indicators of tumor progression, aggressiveness and differentiation. Tumors that are ER positive are associated with a more favorable prognosis. Despite the lack of a documented molecular link between the estrogen receptor and activated Stat3, determining whether their expression associates with expression of activated Stat3 is of interest. Our data has shown a positive correlation between activated Stat3 and ER.

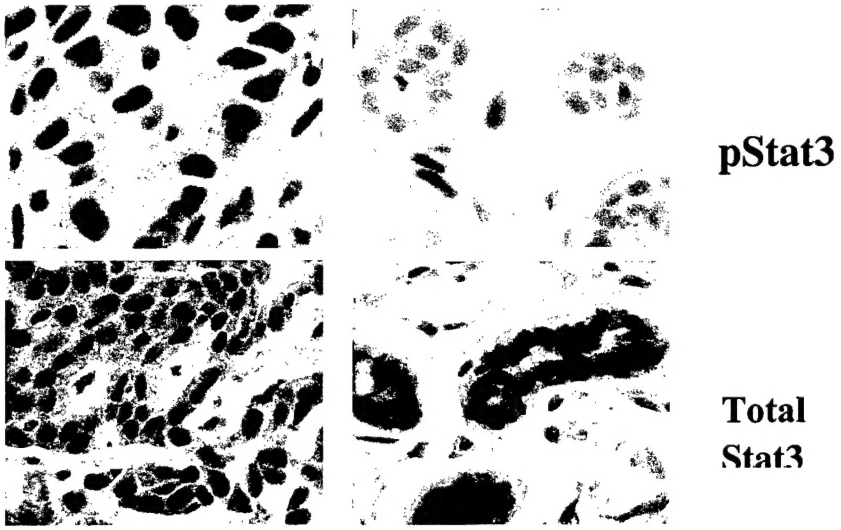

Figure 4. Matched Tumor (on left) and Normal (on right) specimens were stained with anti-phospho Stat3 antibody (top panels) as well as anti-total Stat3 (bottom panel). Normal tissue and cancerous tissue contains abundant total Stat3, while only the Tumor contains high levels of phospho-Stat3.

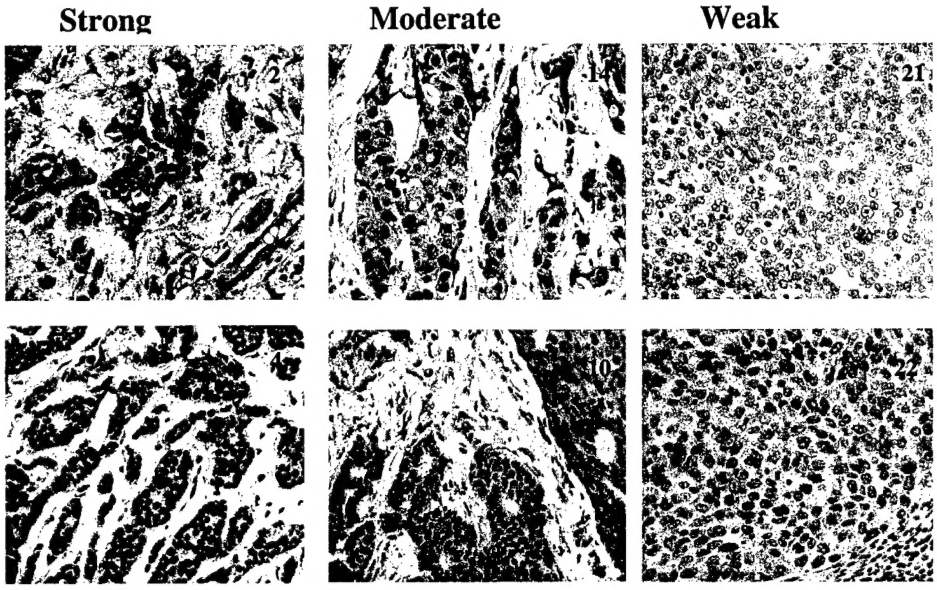

Figure 5. Immunohistochemistry of primary breast cancer samples. 2 examples/>100 for either strong pStat3, moderate pStat3 or weak pStat3 are shown here.

The Her2/neu receptor is a receptor tyrosine kinase that is over-expressed in a number of breast carcinomas and is suggested to be a poor prognostic indicator of breast cancer development. Our data has shown no statistically significant correlation between activated Stat3 and Her2/neu staining. Interestingly, the majority of the negative pStat3 samples were also negative for Her2/neu and ER. Furthermore, we find strong pStat3 staining in pre-invasive tumors including DCIS, LCIS and invasive tumors.

PSTAT3- 9/26 (35%) ER- 9/26 (35%) Her2/neu- 6/26 (23%)

- 66% of the strong pSTAT3 are also ER+ (p=0.002)
- 11% of the strong pSTAT3 are also Her2/neu+ (p=0.5)

We are in the process of staining these samples for EGFR and PDGFR, both of which can lead to the tyrosine phosphorylation of Stat3. Potential downstream target genes of Stat3 include CyclinD1 and Bcl-xl which are critical targets in the development of murine breast cancer. Bcl-xl is not overexpressed in our primary breast cancer specimens, at least when compared to positive control lymphoma and myeloma specimens. We are presently determining Cyclin D1 levels. The negative regulators of Stat3 phosphorylation include SHP-2, TcPTP, SOCS1 and PIAS3. By in situ hybridization we determined that PIAS3 levels were increased rather than decreased in all of the breast cancer samples, suggesting that PIAS3 is not differentially regulated nor does it correlate positively or negatively with strong pStat3 samples (data not shown).

In an attempt to examine the functional role of constitutively activated Stat3 in breast tumorigenesis we targeted Stat3-C to murine breast tissue by using an MMTV driven transgene construct expressing Stat3-C. Unfortuntely, these animals never expressed high levels of Stat3-C nor did they reveal any abnormal phenotype. We have good evidence in a variety of different cell lines that low levels of Stat3-C does not lead to constitutive transcriptional activation or cellular transformation. Our approach to overcome this problem is to generate a model of inducible Stat3-C expression in murine breasts. We are in the process of generating this animal.

Key Research Accomplishments

- Phospho-Stat3 is highly expressed in 35% of primary breast cancer specimens.
- Strong pStat3 staining correlates with ER positivity (statistically significant) and inversely with her2neu staining (not statistically significant).
- Phospho-Stat3 staining by immunohistochemistry requires attention to timing in obtaining samples, as this protein is very labile.
- Bcl-xl, a Stat3 target gene is not upregulated in our breast cancer specimens
- PIAS3, a negative regulator of pStat3 activity, is increased in all breast cancer specimens in contrast to normal tissue.
- MMTV-Stat3-C transgenic mice do not express sufficient levels of Stat3-C to permit any conclusive statements to the effects of this protein on proliferation.

Reportable Outcomes

- We are presently preparing a manuscript which will include much of the above mentioned data.
- I presented this work at 2 meetings this year: 1) Keystone Symposia on Jak/Stat Signaling. Utah, 1/2002. 2) National Endocrine Meeting. San Fransisco 6/2002.
- No patents have been submitted or applied for.
- We are applying for funding from the Susan G. Komen Foundation partly based on the data generated from the data presented here.

Conclusions

Constitutive activation of Stat3 is required for transformation of a number of breast carcinoma derived cell lines and more importantly is sufficient for mediating transformation of immortalized breast epithelial cells. Here we demonstrate that persistently activated Stat3 (pStat3) is highly expressed in ~35% of primary breast cancer specimens as well as pre-invasive DCIS and LCIS. Strong pStat3 levels correlate positively with ER staining and negatively with her2neu. One of the important negative regulators of pStat3, PIAS3 is not transcriptionally down regulated in these specimens.

References

1. J. Bromberg, *J Clin Invest* **109**, 1139-42. (2002).
2. D. E. Levy, J. E. Darnell, *Nat Rev Mol Cell Biol* **3**, 651-62. (2002).
3. R. Garcia, et al., *Cell Growth Differ* **8**, 1267-76 (1997).
4. R. Garcia, et al., *Oncogene* **20**, 2499-513. (2001).
5. J. F. Bromberg, C. M. Horvath, D. Besser, W. W. Lathem, J. E. Darnell, Jr., *Mol. Cell. Biol.* **5**, 2553-8 (1998).
6. J. Bromberg, et al., *Cell* **98**, 295-303 (1999).
7. J. Turkson, et al., *Mol Cell Biol* **18**, 2545-52 (1998).